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TITLE: Fibronectin Matrix Remodeling in the Regulation of the Inflammatory Response within the Lung: An Early Step in Lung Cancer Progression

PRINCIPAL INVESTIGATOR:
Paula J. McKeown-Longo, Ph.D.
Anthony Ambesi, Ph.D.
Carol Horzempa, B.S.
Aparna Prasad, Ph.D.

CONTRACTING ORGANIZATION:
Albany Medical College

Albany, New York 12208-3479

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Paula J. McKeown-Longo, Ph.D.
mckeowp@mail.amc.edu

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14. ABSTRACT

Changes in tissue mechanics as well as increased tissue inflammation have been identified as contributory factors to the development of malignancies. These pathologies are characterized by extensive remodeling of extracellular matrix and increased tissue rigidity. Recent studies have shown that increased tissue rigidity is associated with the unfolding of the Type III domains of extracellular matrix fibronectin. During the previous funding year, we have addressed the hypothesis that changes in secondary structure of fibronectin present in the extracellular matrix drives chronic inflammation within the lung microenvironment. We found that a peptide representing a partially unfolded intermediate of the first Type III repeat of fibronectin (FnIII-1c) induced the expression of cytokines, CXCL1-3, IL-8 and TNF-α, by lung fibroblast cells. The increase in IL-8 expression was dependent on Toll-like receptor 2 and NFκB. Immunohistochemistry of squamous cell carcinoma of the lung revealed extensive stromal staining for IL-8 and fibronectin fibrils which were co-aligned with myofibroblasts. These data suggest that unfolding of FnIII domains secondary to myofibroblast-generated tension induces cytokine expression by stromal fibroblasts. These data implicate changes in fibronectin secondary structure in response to increased tissue rigidity as part of a feed forward mechanism driving chronic inflammation within the lung microenvironment.

15. SUBJECT TERMS
lung cancer, inflammation, fibronectin, cytokine

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INTRODUCTION

Inflammation-mediated destruction of the extracellular matrix, such as that which occurs in chronic obstructive pulmonary disease (COPD) and emphysema, is associated with increased risk of lung cancer. These pathologies are characterized by extensive remodeling of extracellular matrix and changes in the mechanical properties of the tissue. Increased rigidity and loss of tissue compliance often accompany disease progression. The role of tissue mechanics in the initiation and progression of lung cancer is not known. Recent evidence has indicated that the Type III modules of fibronectin present within the matrix can become unfolded as the matrix becomes more rigid (1). As increased tissue rigidity is a common characteristic of both the COPD lung and solid tumors, it seems probable that unfolded Type III fibronectin modules are present within the developing tumor tissue. Evidence also indicates that the exposure of novel biological activities within the unfolded fibronectin Type III repeats may act as mechanosensors transmitting information about the mechanical properties of the matrix back into the cell. Therefore, changes in the mechanical properties of the lung would be expected to be accompanied by changes in the relative unfolding of Type III modules of fibronectin present within the lung extracellular matrix. The impact of this mechanically sensitive information on tumor progression and treatment is virtually unknown. In this study, we used tissue arrays from lung cancer patients and cDNA microarrays representing inflammatory cytokines to test the hypothesis that unfolding of the first Type III repeat of fibronectin regulates stress pathway signaling and subsequent release of inflammatory mediators from lung fibroblast cells present within the lung tumor microenvironment.

BODY

Aim 1: Determine the effect of the fibronectin III-1c peptide on the expression of inflammatory genes by human lung fibroblasts, lung cancer cells, and pulmonary epithelial cells.

FnIII-1c induces inflammatory gene expression in adult lung fibroblasts: The FnIII-1c peptide represents a stable intermediate structure formed during force-induced unfolding of the FnIII-1 domain of fibronectin. When incubated with cultured primary human lung fibroblasts, FnIII-1c induced the expression of several inflammatory cytokines. Microarray analysis (Fig. 1A) identified several inflammatory genes induced in lung fibroblasts by FnIII-1c: IL-8 (CXCL-8), CXCL1-3 and TNF-α were the most highly expressed genes in response to FnIII-1c. Increased expression of IL-8 in response to FnIII-1c was confirmed by ELISA analysis of conditioned medium (Fig. 1B). Another fibronectin Type III domain, FnIII-13, served as negative control. IL-8/CXCL8 and CXCL1, 2, and 3 are pro-angiogenic cytokines known to play important roles in tumor angiogenesis and in neutrophil chemotaxis. These cytokines are recognized by the CXCR2 receptor on endothelial cells and neutrophils. Accumulating data points to the CXCL-CXCR2 signaling axis as a major regulator of lung cancer angiogenesis (2-4). CXCR2 and its ligands, CXCL1, CXCL8 (IL-8), have been proposed as important alternative angiogenic regulators to the VEGF/VEGFR axis. Recent preclinical and clinical models have shown that resistance to the anti-angiogenic drug, bevacizumab, is mediated by CXCL8/IL-8 (5). CXCR2 has been implicated in the progression of lung inflammation and fibrotic disease (6,7). Our studies are the first to demonstrate that the release of these cytokines from stromal cells can be regulated by changes in the secondary structure of fibronectin.

FnIII-1c induces TLR2-dependent expression of IL-8 in human lung fibroblasts. Induction of inflammatory cytokines is often regulated by the activation of the transcription factor, NFκB which is translocated to the nucleus. NFκB dependent expression of cytokines is often regulated through Toll-like receptors (TLRs). To determine a role for NFκB in the induction of IL-8 in response to FnIII-1c, cells were preincubated with two different inhibitors of NFκB activation, PS-1145 and BAY-7082. Both of these inhibitors blocked IL-8 secretion in response to FnIII-1c (Fig. 1B). The effects of FnIII-1c on IL-8 expression were dose-dependent (Fig. 2A) and accompanied by the nuclear translocation of the RelA/p65 subunit of NFκB (Fig. 2B). As the TLR family of receptors are known to mediate the NFκB dependent induction of cytokines and to be expressed on lung tissue cells, we used blocking antibodies to TLR2 and TLR4 to evaluate their potential role in the induction of cytokines in response to FnIII-1c. As shown in Fig. 2C, preincubation of cells with a blocking antibody to TLR2 resulted in a dose-dependent inhibition of IL-8 secretion in response to FnIII-1c. In contrast, blocking the TLR4 receptor had no effect on IL-8 expression.

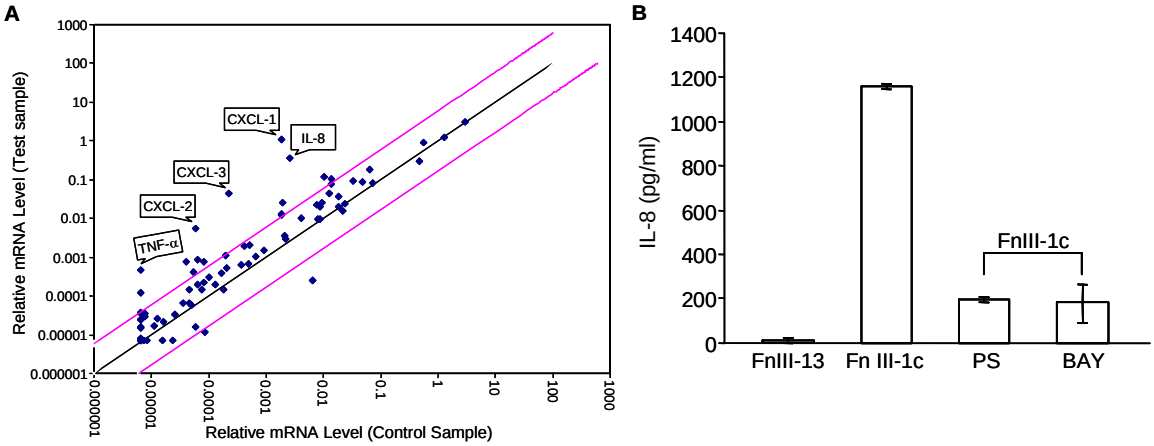

Fig. 1. *III-1c induces expression of inflammatory genes in lung fibroblasts.* **(A)** Monolayers of human fibroblasts from adult lung were treated with 10 μM FnIII-1c or PBS in serum-free culture medium supplemented with 0.1% BSA for 2 hrs. Expression profile of genes was performed using the cytokine and inflammation PCR Array (SABio-sciences). Pink lines indicate a 5-fold change in baseline. Five inflammatory genes, as designated by flags, were highly upregulated in response to FnIII-1c. (**B**) Induction of IL-8 by FnIII-1c is dependent on NFκB . Monolayers of human lung fibroblasts were serum starved overnight and then pretreated with inhibitors of NFκB signaling. The inhibitors, 10 μM of BAY11-7082 or 10 μM of PS-1145 were added 2 hrs prior to the addition of 10μM of FnIII-1c for an additional 4 hrs. Control cells were treated with FnIII-13. Conditioned medium from cells was collected and IL-8 was measured by ELISA.

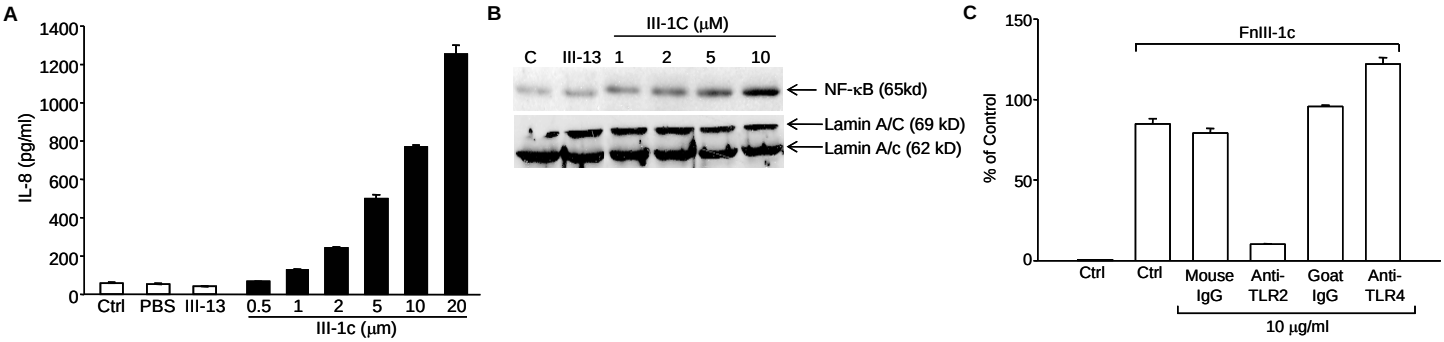

Fig. 2. FnIII-1c induces TLR2-dependent expression of IL-8 in human adult lung fibro- blasts. (**A**) Human adult lung fibroblasts were treated with increasing concentrations of FnIII-1c or FnIII-13 for 4 hrs. IL-8 concentration in culture supernatant was determined by ELISA. (**B**) FnIII-1c not FnIII-13 induced the nuclear translocation of NFκB. Human adult lung fibroblasts were incubated with the indicated concentrations of III-1c with PBS for 1 hr. Nuclear fraction was isolated and analyzed by Western blotting for the presence of the NFκB protein, p65/rel A. Blots were stripped and reprobed with antibody against nuclear lamin A/C as a loading control. (**C**) Induction of IL-8 in human lung fibroblasts by FnIII-1c is dependent on TLR-2. Human adult lung fibroblasts were serum starved overnight in 0.1% BSA/HLF medium and pretreated with increasing concentrations of antibodies to human TLR-2 or control mouse IgG for 1 hr prior to the addition of 20 μM FnIII-1c for 4 hrs. IL-8 present in the conditioned medium was analyzed by ELISA. Error bars represent mean ± SD.

FnIII-1c activates NFκB and MAP kinase pathways in human lung cancer cells. FnIII-1c also stimulated the release of IL-8 from two non-small cell lung cancer cell lines (Calu-1 and H460) (Fig. 3A). In Calu-1 cells, FnIII-1c induced a 10-fold increase in IL-8 levels within 3 hrs. A similar but lower (2.5 fold) increase in IL-8 induction was seen in H460 cells, but the overall levels of IL-8 in these cells was much higher (9 ng/ml vs 2.0 ng/ml) than that seen in Calu-1 cells (Fig. 3A). Addition of FnIII-1c to human lung cancer cells, Calu-1, resulted in the activation (nuclear translocation) of NFκB as well as the phosphorylation of p38 and JNK MAP kinases (Fig. 3B-D). Taken together, the data in Figs. 1-3 suggest that FnIII-1c induces the activation of signaling pathways leading to the release of inflammatory cytokines from cells within the lung tumor microenvironment.

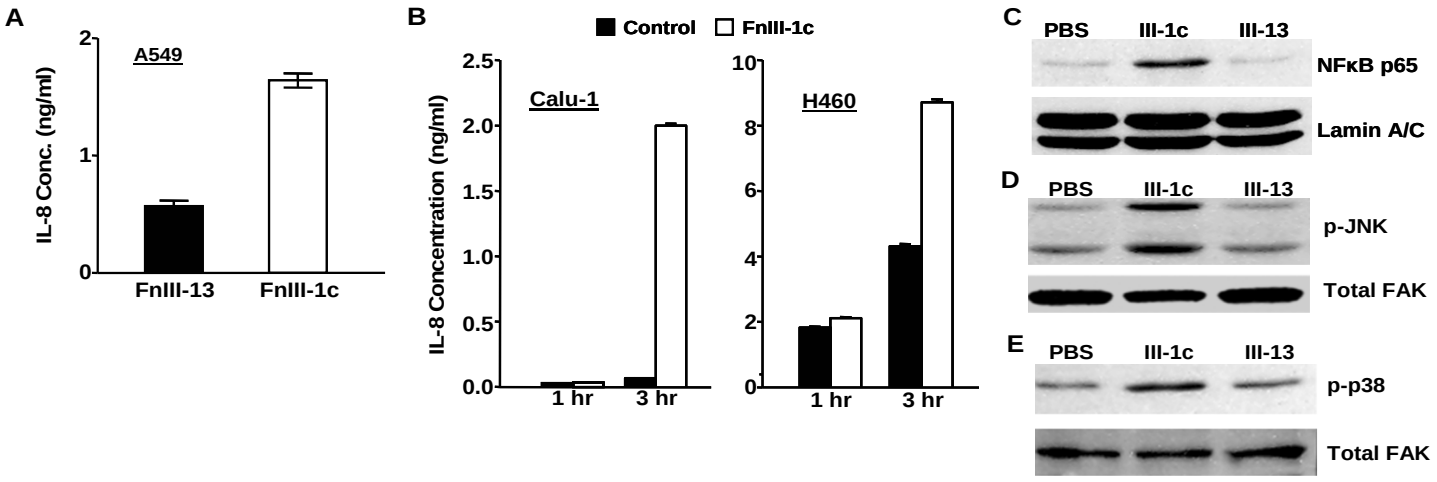

Fig. 3. *FnIII-1c induces IL-8 and activates NFκB and MAP kinases in human non small cell lung cancer cells.* (**A**) Calu-1 and H-460 cell monolayers were incubated with 10 μM FnIII-1c for the designated times and the conditioned medium was assayed for IL-8 by ELISA. Bars indicate standard error of the mean for triplicate samples. (**B**) Calu-1 human lung cancer cells were treated with 20 μM FnIII-1c, FnIII-13 or PBS in 0.1% BSA/DMEM for 1 hr. (**C**) The nuclear fraction was isolated and analyzed by Western blot for the presence of the NFκB protein P65/relA. The membranes were then stripped and reprobed with an antibody against nuclear Lamin A/C as loading control. (**C,D**) The cytosolic fraction was electrophoresed and immunoblotted using antibodies against phosphorylated JNK, or phosphorylated p38. The membranes were then stripped and reprobed with antibodies against FAK as loading control.

Aim 2: Determine the effect of substrate rigidity on fibronectin-dependent changes in inflammatory gene expression by lung tumor cells.

The findings in Aim 1 suggested that changes in fibronectin secondary structure could regulate cytokine release from stromal fibroblasts as well as lung cancer cells. To validate this observation in vivo, we undertook a study to determine whether evidence for such a pathway existed in human tumors.

Fibronectin is preferentially expressed in the stroma of human lung tumors. Tissue microarrays containing samples of human squamous cell carcinoma of the lung were stained for fibronectin using the 9D2 monoclonal anti-body which recognizes an epitope within the III-1 domain of fibronectin. Figure 4 shows heavy staining for fibronectin in two samples of squamous cell carcinoma (**A, B**) in the stroma (stroma) adjacent to the infiltrating border of carcinoma cells (tumor). The stroma shows extensive fibrillar staining for fibronectin with aligned fibroblasts (F) and infiltrating inflammatory cells (I). There was also diffuse staining for fibronectin throughout the tumor, particularly in Sample 2 (**B**). Fibronectin staining was less pronounced in normal lung tissue (**C**). These data suggest that fibronectin is heavily expressed within the stroma of human lung cancers and that epitopes within the FnIII-1 domain are accessible within these tissues.

The stroma of human lung tumors is enriched for myofibroblasts and IL-8. Fig. 5 shows a section of squamous cell carcinoma of the lung stained using a monoclonal antibody to either smooth muscle cell actin (SMA) (Fig. 5A) or IL-8 (Fig. 5B). In Fig. 5A, the tumor stroma stained heavily for SMA and staining patterns were similar to those seen for fibronectin (Fig. 4A), suggesting that the fibroblasts aligned along fibronectin fibrils are myofibroblasts. Fig. 5B shows moderate to heavy staining for IL-8 in both the stroma and tumor. Figure 5C is a negative control for non-specific staining. Taken together, the data shown in Figs. 4 and 5 indicate that in squamous cell carcinoma both fibronectin and IL-8 are heavily expressed in the tumor stroma and that the increase fibronectin in the stroma is associated with an influx of myofibroblasts which appear to be aligned along fibronectin fibers. These data are consistent with our hypothesis and suggest that myofibroblast generated contractility may unmask cryptic sites within the secondary structure of fibronectin, which then initiate signaling pathways leading to the release of inflammatory cytokines within the lung tumor microenvironment.

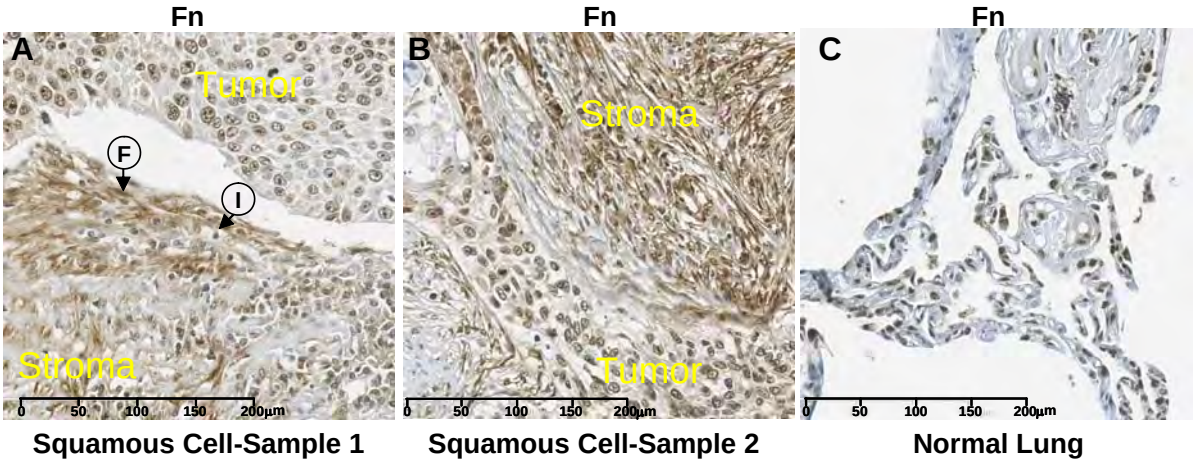

Fig. 4. Panel **A** shows a section of a squamous cell carcinoma of the lung, which has been stained for fibronectin with the monoclonal antibody, 9D2, which recognizes the first Type III domain of fibronectin. The top of the panel shows the infiltrating tumor. The lower half shows the tumor stroma which stains heavily for fibronectin. The fibronectin appears fibrillar and is associated with fibroblasts (F). The stroma also shows extensive infiltration with Immune cells (I). Panel **B** shows a second example of a lung squamous cell carcinoma. The stroma, shown in the top right is heavily stained for fibrillar fibronectin. The tumor shows moderate staining for fibronectin. Panel **C** shows normal lung tissue which exhibits less pronounced staining for fibronectin.

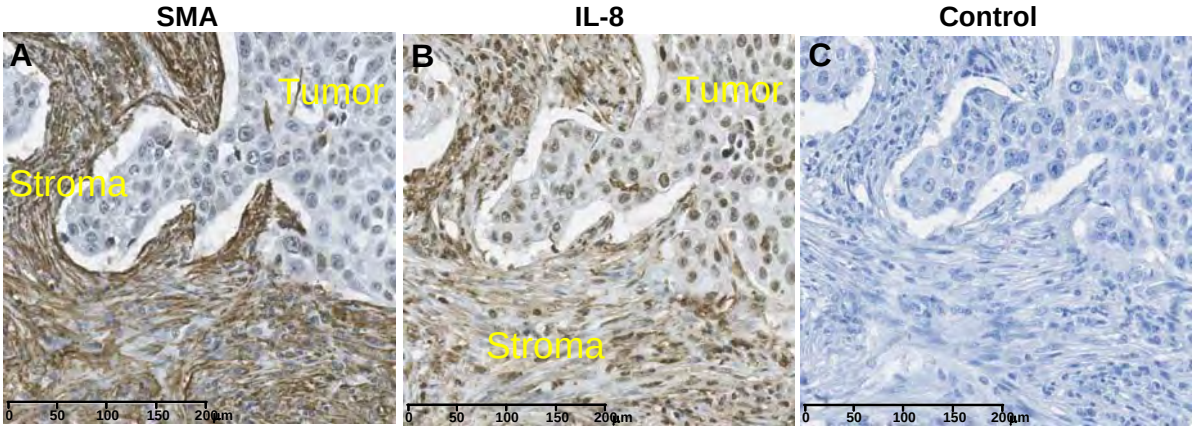

Fig. 5. Lung tumor stroma is enriched for IL-8 and myofibroblasts. A sample of squamous cell carcinoma was stained for IL-8 and smooth muscle actin (SMA) using monoclonal antibodies. **A**) Heavy staining for SMA is seen within the stroma indicating the presence of myofibroblasts. **B**) Both stroma and tumor stained positively for IL-8. **C**) Control tissue stained with only secondary antibody is negative.

Fibronectin fragments containing the First Type III domain are present in lung tumor tissue. To determine whether FnIII-1 containing fibronectin fragments were present in lung tumors, we analyzed lysates from 6 lung tumors for the presence of fragments containing the FnIII-1 domain. Lysates were analyzed by Western blot using the 9D2 antibody which recognizes the carboxyl half of FnIII-1. As shown in Fig. 6, all 6 samples from a variety of tumor specimens showed a wide range of III-1c containing fibronectin fragments ranging from 230 kDa (intact fibronectin) to 24 kDa (2 Type III domains). The data suggest that biologically active regions of fibronectin may also be unmasked in response to proteolysis.

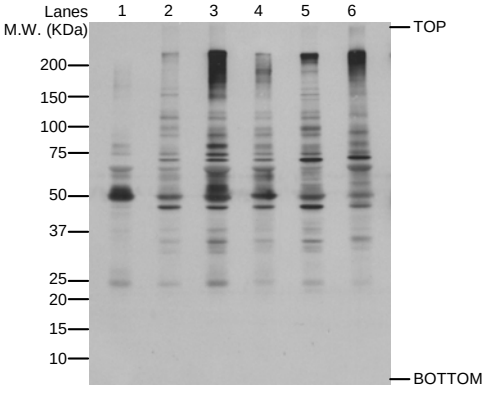

Fig. 6. Human lung tumor tissue lysates were separated on a 6-14% gradient polyacrylamide gel under reducing condition and processed for Western blot with anti-FnIII-1 mAb 9D2. The diagnosis of those tumor samples are:

Lane 1: adenocarcinoma, grade 3, stage IB
Lane 2: adenocarcinoma, grade 2, stage I
Lane 3: squamous cell carcinoma, grade 2, stage IIIA
Lane 4: squamous cell carcinoma, grade 2, stage I
Lane 5: squamous cell carcinoma, grade 3, stage IIIA
Lane 6: adenocarcinoma, grade 2, stage II

KEY RESEARCH ACCOMPLISHMENTS

- ►An unfolded intermediate from the first Type III repeat of fibronectin (FnIII-1c) activates an NFκB/TLR2 dependent signaling pathway in adult lung fibroblasts which induces the expression of pro-angiogenic cytokines, CXCL1-3, IL-8, TNF-α.

- ►FnIII-1c induces IL-8 expression in lung cancer cells (Calu, H460, A549).

- ►Fn is strongly expressed within lung tumor stroma and epitopes within the FnIII-1 domain are accessible within the tissue.

- ►Fn and IL-8 co-localized with myofibroblasts within the stroma of squamous cell carcinoma of the lung.

REPORTABLE OUTCOMES

Manuscript:

Zheng, M., Jones, D.M., Horzempa, C., Prasad, A., and McKeown-Longo, P.J.: The first type III domain of fibronectin is associated with the expression of cytokines within the lung tumor microenvironment. J Cancer 2:478-483, 2011.

Funding applied for based on work supported by this award:

DoD - Lung Cancer Research Program (LCRP) - Investigator-Initiated Translational Research Award - Letter of Intent - Pre-application was not invited for full application submission.

NIH - R01- Fibronectin Matrix Remodeling in Lung Cancer Progression - Dates of Project: 04/01/12-03/31/17 - currently under review.

CONCLUSION

In summary, our preliminary data show that FnIII-1c, a peptide representing a stable intermediate structure predicted to occur during force dependent unfolding of fibronectin, induces the expression of inflammatory cytokines in lung tumor cells. Cytokine induction by FnIII-1c in fibroblasts and lung tumor cells is dependent on TLR2 and NFκB. Staining of human lung tumors confirms the presence of fibronectin matrix and aligned myofibroblasts within the tumor stroma suggesting that stromal fibronectin is subjected to myofibroblast generated contractile forces. Consistent with a role for fibronectin in mediating cytokine expression within the tumor microenvironment, the lung tumor stroma is also enriched for IL-8. These data support our hypothesis that force dependent unfolding of fibronectin in the tumor stroma drives an inflammatory response within the lung tumor microenvironment. The identification of mechanically sensitive response elements within the fibronectin matrix, and their associated signaling pathways will provide novel targets for the treatment of chronic lung inflammation and may also represent a unique opportunity for developing therapies directed at early stage intervention in that population (i.e., former smokers, patients with COPD) who are at high risk for the development of lung cancer.

REFERENCES

1. Antia M, Baneys G, Kubow KE, Vogel V. Fibronectin in aging extracellular matrix fibrils is progressively unfolded by cells and elicits an enhanced rigidity response. Farady Discuss. 2008; 139:229-49.
2. Keane MP, Belperio JA, Xue YY, Burdick MD, Strieter RM. Depletion of CXCR2 inhibits tumor growth and angiogenesis in a murine model of lung cancer. J Immunol. 2004; 172:2853-60.
3. Martin D, Galisteo R, Gutkind JS. CXCL8/IL8 stimulates vascular endothelial growth factor (VEGF) expression and the autocrine activation of VEGFR2 in endothelial cells by activating NFκB through the CMB (Carma3/Bcl10/Malt1) Complex. J Biol Chem. 2009; 284:6038-42.
4. Yanagawa J, Walser TC, Zhu LX, Hong L, Fishbein MC, Mah V et al. Snail promotes CXCR2 ligand-dependent tumor progression in non-small cell lung carcinoma. Clin Cancer Res. 2009; 15:6820-9.
5. Schultheis AM, Lurje G, Rhodes KE, Zhang W, Yang D, Garcia AA et al. Polymorphisms and clinical outcome in recurrent ovarian cancer treated with cyclophosphamide and bevacizumab. Clin Cancer Res. 2008; 14:7554-63.
6. Russo RC, Guabiraba R, Garcia CC, Barcelos LS, Roffe E, Souza AL et al. Role of the chemokine receptor CXCR2 in bleomycin-induced pulmonary inflammation and fibrosis. Am J Respir Cell Mol Biol. 2009; 40:410-21.
7. Barnes PJ. New therapies for chronic obstructive pulmonary disease. Med Princ Pract. 2010; 19:330-8.

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Short Research Communication

The First Type III Domain of Fibronectin is Associated with the Expression of Cytokines within the Lung Tumor Microenvironment

Mingzhe Zheng[1], David M. Jones[2], Carol Horzempa[1], Aparna Prasad[1], and Paula J. McKeown-Longo[1] ✉

1. Center for Cell Biology and Cancer Research, Albany Medical College, Albany, New York, USA
2. Department of Pathology, Albany Medical College, Albany, New York, USA

✉ Corresponding author: Paula J. McKeown-Longo, Ph.D., Center for Cell Biology and Cancer Research, Mail Code 165, Albany Medical College, 47 New Scotland Avenue, Albany, NY 12208. Telephone: (518) 265-5651; Fax: (518) 262-5669; E-mail: mckeowp@mail.amc.edu

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Abstract

Recent studies have pointed to changes in tissue mechanics as a contributory element to the development of malignancies. Increased tissue rigidity is associated with the unfolding of the Type III domains of fibronectin within the extracellular matrix. The consequences of this unfolding on cellular functions within the lung are not well understood. In the present study, we evaluated the effect of a peptide representing a partially unfolded intermediate of the first Type III repeat of fibronectin (FnIII-1c) on inflammatory gene expression in adult human lung fibroblast cells. FnIII-1c induced expression of cytokines, CXCL1-3, IL-8 and TNF-α, by lung fibroblast cells. The increase in IL-8 expression was dependent on Toll-like receptor 2 and NFκB. Immunohistochemistry of tissue arrays representing squamous cell carcinoma of the lung revealed extensive stromal staining for IL-8 and fibronectin fibrils which were co-aligned with myofibroblasts. These data suggest a model in which unfolding of FnIII domains secondary to myofibroblast-generated tension may induce the release of cytokines by stromal fibroblasts present within the lung tumor.

Key words: lung cancer, inflammation, fibronectin, cytokine

INTRODUCTION

Lung cancer is the leading cause of cancer-related death with a world-wide annual death rate of 1.3 million people (1). In spite of developments in surgical techniques, chemotherapy and radiation oncology, the overall 5 year survival rate (15%) has remained unchanged for decades. Inflammation mediated destruction of the extracellular matrix, such as that which occurs in chronic obstructive pulmonary disease (COPD), asthma, tuberculosis and emphysema, is associated with increased risk of lung cancer (2-4). The association between lung inflammatory disease and lung cancer has been recognized for several decades, however, the mechanisms underlying their linkage are not understood. Recent studies have suggested that chronic inflammation and extracellular matrix remodeling may provide a mechanistic link between COPD and the development of lung cancer (2).

Increased deposition of plasma fibronectin into the lung extracellular matrix is seen in lung cancer and has been implicated in promoting tumor cell proliferation, invasion and resistance to chemotherapy (5-10). Changes in the mechanical properties of lung tissue are seen in a number of disease states including cancer, COPD, asthma and emphysema, where changes in the remodeling and stiffening of the extracellular matrix accompany disease progression. A recent study of lung fibrosis in bleomycin treated mice, has shown that early fibrotic lesions in the lung are associated with large (as much as 30 fold) changes

in the lung tissue stiffness. These large increases in tissue rigidity occur in focal areas in association with extracellular matrix remodeling and infiltration of myofibroblasts (11). The role of tissue stiffening in the initiation and progression of lung cancer has not been studied, but studies on breast cancer show that increased tissue stiffening is positively correlated with disease progression (12).

The secondary structure of the fibronectin molecule is organized into individually folded domains termed Types I, II and III. The Type III domains are folded into structurally stable beta-pleated sheets which unfold and refold in response to changes in intracellular tension. The biological role of many of these domains is not well understood, but accumulating evidence has indicated that the Type III domains of fibronectin present within the matrix become unfolded as the matrix becomes more rigid (13).

Steered molecular dynamics has been used to predict stable intermediate structures of partially unfolded fibronectin Type III domains. One such intermediate is recapitulated in a peptide comprised of the C-terminal two-thirds of the first FnIII domain, FnIII-1c (14). In the present study, we show that addition of FnIII-1c to adult lung fibroblasts causes a large increase in the expression of inflammatory cytokines including CXCL1-3 and IL-8. Staining of tissue microarrays of squamous cell carcinoma shows extensive myofibroblast infiltration into a fibronectin rich stroma coupled with increased expression of IL-8. Our studies are consistent with a model in which myofibroblast driven unfolding of the secondary structure of matrix fibronectin plays a role in the initiation and/or maintenance of chronic inflammation within the tumor microenvironment.

MATERIALS AND METHODS

Analysis of gene expression

Primary human adult lung fibroblasts were grown in lung/cardiac fibroblast growth medium (Cell Applications, Inc. San Diego, CA) and used from passage 3-6. In most experiments, cell monolayers were serum-starved overnight with serum-free medium (lung/cardiac fibroblast basal medium containing 0.1% BSA) before treatment. Recombinant His-tagged fibronectin modules, III-1c and III-13, were prepared as described previously (15). Levels of contaminating endotoxin (<0.25 units/nmole of protein) in recombinant proteins were determined using the limulus amebocyte lysate assay, QCL-1000 (Lonza, Walkersville, MD). Total RNA was isolated using RNAeasy Plus Mini Kit (Qiagen, Valencia, CA). The RT² First Strand Kit was used to convert RNA into first strand cDNA. Mixtures of cDNA and SYBR® Green qPCR Mastermix were applied to the Human Inflammatory Response and Autoimmunity PCR Array Plates (SABiosciences, Frederick, MD). A MyiQ cycler system (Bio-Rad Laboratories) was used for real-time PCR detection. The data were analyzed using the Excel-based PCR array data analysis templates provided by the manufacturer. The inhibitors of NFκB signaling PS-1145 (Sigma-Aldrich) and BAY 11-7082 (Calbiochem) were dissolved in DMSO. IL-8 protein concentration was measured by ELISA (ELISA Set from BD Biosciences, San Diego, CA). Blocking antibodies to human TLR2 and TLR4 were from R&D Systems (Minneapolis, MN).

Tissue section staining

Non-small cell human lung carcinoma tissue with self-matching normal adjacent tissue microarray panels (US Biomax Inc, Rockville, MD), were processed for immuno-staining using peroxidase-based ABC system (Vector Laboratories, Burlingame, CA). The primary antibodies and their dilution were: 10 μg/ml IgG of anti-human FnIII-1 mouse monoclonal antibody 9D2, 25 μg/ml IgG of anti-human IL-8 mouse monoclonal antibody (R&D System, Minneapolis, MN) or 1:1600 dilution ascites of anti-α smooth muscle actin mouse monoclonal antibody (A2547, Sigma, St. Louis, MO). Color was developed by reaction with 3,3'-Diaminobenzidine. Tissue sections were counterstained with hematoxylin.

RESULTS

FnIII-1c induces the expression of inflammatory cytokines in adult lung fibroblasts

To determine the effect of FnIII domain unfolding on gene expression, we incubated human adult lung fibroblast cells with the FnIII-1c peptide. This peptide recapitulates a stable intermediate structure which is formed when fibronectin undergoes unfolding in response to cellular force (14). After 2 hours of incubation with FnIII-1c, RNA was extracted from cells and gene expression was analyzed by microarray. As shown in Fig. 1A, several genes were upregulated in response to FnIII-1c and the five most highly upregulated genes (CXCL-1,2,3, IL-8 and TNF-α) are shown in Fig. 1B. IL-8, which exhibited over a 100 fold increase in expression in response to FnIII-1c was chosen for further characterization. The microarray results for IL-8 were confirmed by ELISA, which demonstrated a dose-dependent increase in the amount of IL-8 protein present in the conditioned medium of lung fibroblasts treated with FnIII-1c. The

FnIII-13 domain served as control and had no effect on IL-8 expression (Fig. 1C).

FnIII-1 induction of IL-8 requires TLR-2 and NFκB

Expression of inflammatory cytokines is often under the control of the transcription factor, NFκB, which is translocated to the nucleus following activation. To determine whether FnIII-1c activated NFκB, nuclear lysates were prepared from lung fibroblasts treated with increasing doses of FnIII-1c. Western blot analysis of lysates indicated that within an hour of FnIII-1c treatment, there was a dose dependent increase in the accumulation of the p65/relA subunit of NFκB in the nucleus (Fig. 2A). To verify a role for NFkB in the induction of IL-8, cells were pretreated with two different inhibitors of NFκB activation, PS-1178 and BAY11-7072. Incubation of cells with FnIII-1c resulted in a large increase in the amount of IL-8 present in the conditioned medium when compared with the control, FnIII-13 (Fig. 2B). In the presence of either inhibitor, FnIII-1c induction of IL-8 was decreased by 85%, confirming a role for NFkB in the induction of IL-8 by FnIII-1c. Activation of NFκB can be regulated by the Toll-like family of receptors (TLRs), transmembrane receptors which regulate the innate immune system. To evaluate a role for TLRs in the induction of IL-8, cells were incubated with FnIII-1c in the presence of blocking antibody to either TLR2 or TLR4. Blocking antibody to TLR2 inhibited IL-8 expression in response to FnIII-1c (Fig. 2C). Neither control antibodies nor blocking antibody to TLR4 inhibited IL-8 secretion in response to FnIII-1c. Taken together, these experiments indicate that the induction of IL-8 expression in lung fibroblasts by FnIII-1c occurs through the TLR2 dependent activation of NFκB.

Fibronectin is preferentially expressed in the stroma of human squamous cell carcinoma

Tissue microarrays containing samples of squamous cell carcinoma of the lung were stained for fibronectin using the 9D2 monoclonal antibody to fibronectin which recognizes an epitope within the FnIII-1 domain (16). Fig. 3A shows heavy staining for fibronectin in two samples of squamous cell carcinoma (panels a and b) in the stroma (stroma) adjacent to the infiltrating border of carcinoma cells (tumor). The stroma show extensive fibrillar staining for fibronectin with aligned fibroblasts (F) and infiltrating inflammatory cells(I). There was also light to moderate diffuse staining for fibronectin within the tumor. Staining for fibronectin in normal lung tissue was less pronounced (panel c).

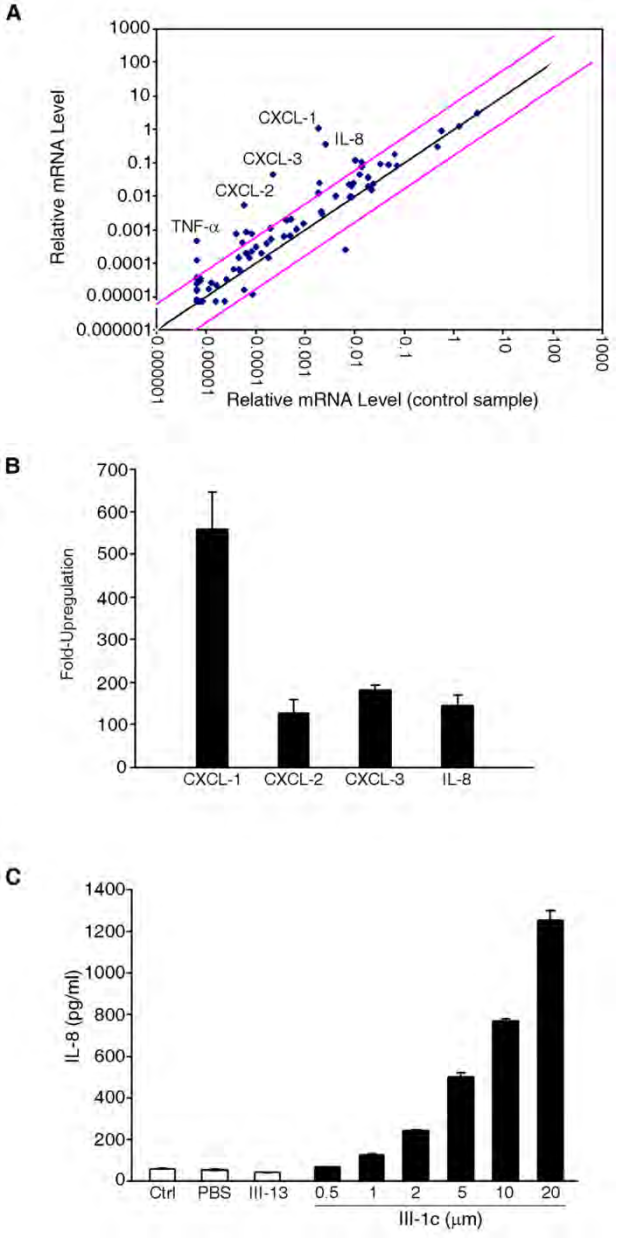

Figure 1. FnIII-1c induces expression of inflammatory genes in lung fibroblasts. (A) Monolayers of adult lung human fibroblasts were treated with 10 μM FnIII-1c or PBS in serum-free culture medium supplemented with 0.1% BSA for 2 hrs. Expression profiling of genes was performed using the Human Inflammatory Response PCR Array. Pink lines indicate a 5-fold change in baseline. Five inflammatory genes (labeled) were highly upregulated in response to FnIII-1c. (**B**) The fold induction of inflammatory genes by FnIII-1c is shown. Values represent the average fold increase over housekeeping genes from 3 separate experiments. (**C**) Human adult lung fibroblasts were treated with increasing concentrations of FnIII-1c or FnIII-13 for 4 hrs. IL-8 concentration in the culture medium was determined by ELISA. Error bars represent mean ± SD.

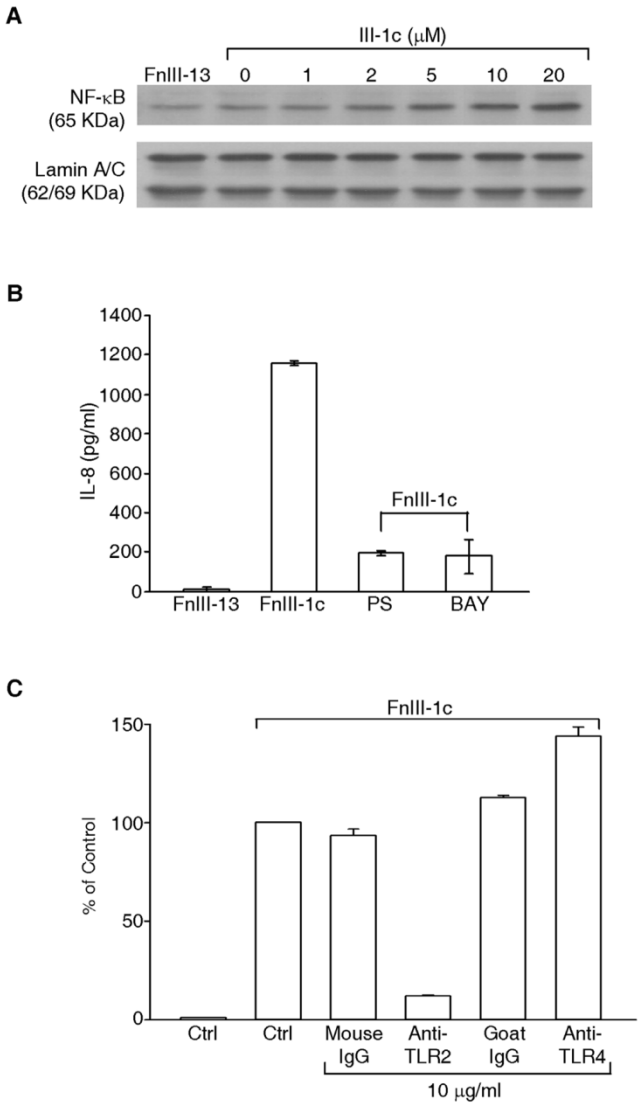

Figure 2. FnIII-1c induces TLR2-dependent expression of IL-8 in human adult lung fibroblasts. (**A**) Human adult lung fibroblasts were incubated with the indicated concentrations of FnIII-1c with PBS for 1 hr. The nuclear fraction was isolated and analyzed by immunoblotting for the presence of the NFκB protein, p65/rel A. Nuclear lamin A/C served as a loading control. (**B**) Cell were pretreated for 2 hrs with inhibitors of NFκB signaling, 10 μM of BAY11-7082 or 10 μM of PS-1145. Cell then received 10 μM FnIII-1c for FnIII-13 for an additional 4 hrs. Conditioned medium from cells was collected and IL-8 was measured by ELISA. (**C**) Cells were pretreated with blocking antibodies to human TLR2, TLR4, or control IgG for 1 hr prior to the addition of 10 μM FnIII-1c for 4 hrs. IL-8 present in the conditioned medium was analyzed by ELISA. Positive control was set as 100%. Error bars represent mean ± SD.

The stroma of human lung tumors is enriched for myofibroblasts and IL-8

Figure 3B shows sequential sections of a squamous cell carcinoma of the lung which have been stained for either smooth muscle cell actin (panel a) or IL-8 (panel b). The tumor stroma (stroma) stained heavily for smooth muscle actin and the staining pattern appeared fibrillar, similar to that seen with fibronectin (Fig. 3A), indicating that the fibroblasts aligned along the fibronectin fibrils are myofibroblasts. IL-8 staining was present in both the stroma and the carcinoma regions of the tumor (panel b). Control staining was negative (panel c). These data indicate that in squamous cell carcinoma both fibronectin and IL-8 are heavily expressed in the tumor stroma and that the increased fibronectin within the tumor stroma is associated with an influx of myofibroblasts which are aligned along fibronectin fibers. These data are consistent with a model in which myofibroblast contractility dependent remodeling of fibronectin matrix initiates the release of inflammatory cytokines within the lung tumor microenvironment.

DISCUSSION

Chronic inflammation and fibrotic disease are believed to be contributing factors in the progression of lung cancer in high-risk patients (2,3). Our data are consistent with a model in which changes in the secondary structure of matrix fibronectin driven by increased rigidity of lung tissue is part of a feed forward mechanism controlling the release of inflammatory mediators into the lung microenvironment. The cytokines released in response to FnIII-1c, IL-8, CXCL1, 2, and 3, are CXCR2 ligands which promote both inflammation and angiogenesis through the recruitment of inflammatory cells and the mobilization of bone marrow endothelial cells (17,18). Our data suggest that unfolding of the FnIII-1 domain within fibronectin present in the tumor stroma promotes tumor progression through the release of cytokines from resident lung fibroblasts.

The FnIII-1c peptide used in this study was originally called anastellin and shown to have anti-angiogenic and anti-tumor properties (19). We subsequently showed that FnIII-1c had inhibitory effects on signaling pathways regulating endothelial microvessel cell proliferation (20,21). In contrast, when incubated with fibroblast cells, FnIII-1c had little effect on growth and instead activated signaling pathways leading to the release of cytokines, which are known to be proangiogenic (20,22). These data suggest that the FnIII-1 domain of fibronectin may have both pro- and anti-angiogenic roles within the tumor microenvironment.

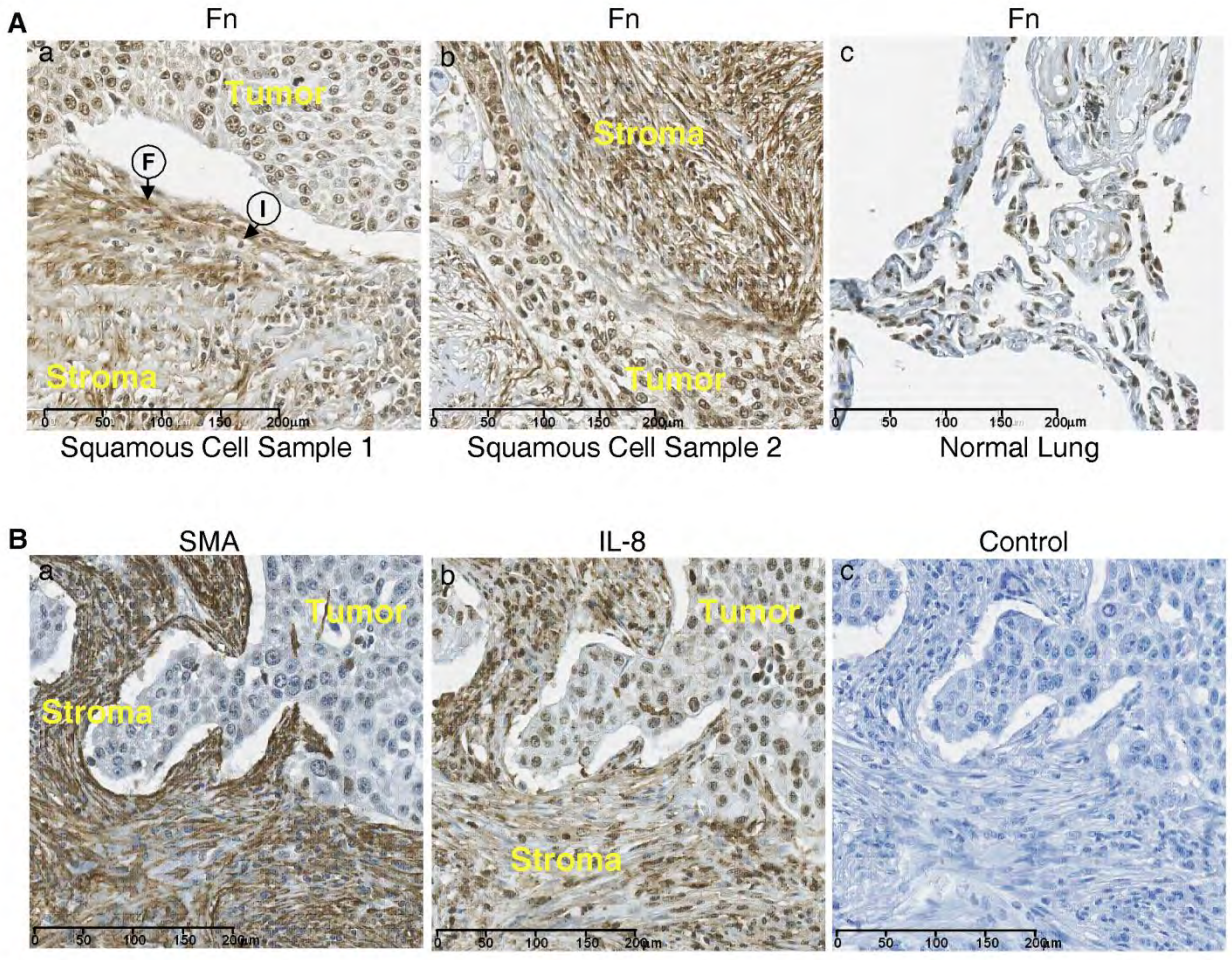

Figure 3. The stroma of squamous cell carcinoma is enriched in fibronectin. (A) Tissue sections from two lung squamous cell carcinomas (panels a and b) and a section of normal lung tissue (panel c) were stained for fibronectin . Fibroblasts (F) and Immune cells (I) are present. (**B**) Sequential sections of squamous cell carcinoma were stained for IL-8 (panel a) and smooth muscle actin (SMA) (panel b). Control tissue stained with only secondary antibody (panel c).

Although the function of TLRs in the regulation of inflammation in response to bacterial pathogens has been recognized for many years, more recent studies have pointed to a role of TLRs in mediating non-septic inflammatory responses. It is now becoming clear that TLRs may play a role in the initiation and/or maintenance of inflammatory processes during organ dysfunction and tissue injury, thereby contributing to chronic illness. Both TLR2 and TLR4 are expressed in resident lung cells and both TLR2 and TLR4 have been reported to be activated through endogenous molecules including fragments of matrix proteins, such as hyaluronic acid (HA), the EDA domain of fibronectin (FnEDA) as well as heat shock proteins and β-amyloid (23,24). Accumulating evidence indicates that TLR signaling is exceedingly complex and the elicited immune response is both ligand- and cell-type specific (25).

We have shown in a previous study, that addition of Fn III-1c to human dermal fibroblasts results in the NFkB dependent release of inflammatory mediators, including IL-8 (22). In dermal fibroblasts, induction of cytokine expression was dependent on TLR4 rather than TLR2. The basis for the involvement of distinct TLR receptors in the two cell types is not known. The ability of FnIII-1c to signal through either TLR2 or TLR4 suggests that FNnIII-1c does not bind directly to TLRs to initiate signaling, but instead interacts with TLR signaling complexes by means of a co-receptor or ancillary binding protein.

The assembly of TLR containing signaling complexes which differentially function to regulate

unique patterns of cytokine expression suggests that it may be possible to selectively target arms of TLR signaling pathways to treat disease without sacrificing pathways controlling host-defense. Our data suggest that force-dependent remodeling of the fibronectin matrix promotes inflammation with the lung. The identification of mechanically sensitive response elements within the fibronectin matrix, and their associated signaling pathways will provide novel targets for the treatment of chronic lung inflammation and may also represent a unique opportunity for developing therapies directed at early stage intervention in that population (i.e., former smokers, patients with COPD) who are at high risk for the development of lung cancer.

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CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

REFERENCES

1. Jemal A, Siegl R, Ward E, Hao Y, Xu J, Murray T et al. Cancer statistics, 2008. CA Cancer J Clin. 2008; 58(2):71-96.
2. Houghton AM, Mouded M, Shapiro SD. Common origins of lung cancer and COPD. Nature Med. 2008; 14:1023-4.
3. Mackinnon AC, Kopatz J., Sethi T. The molecular and cellular biology of lung cancer: identifying novel therapeutic strategies. Brit Med Bulletin. 2010; 95(47):61.
4. Lee G, Walser TC, Dubinett SM. Chronic inflammation, chronic obstructive pulmonary disease, and lung cancer. Current Opin Pulmon Med. 2009; 15:303-7.
5. Liu G, Meng X, Jih Y, Bai J, Zhao Y, Cui X et al. Inhibitory role of focal adhesion kinase on anoikis in the lung cancer cell A549. Cell Biol Int. 2008; 32:663-70.
6. Jia D, Yan M, Wang X, Liang L, Liu L, Kong H et al. Development of a highly metastatic model that reveals a crucial role of fibronectin in lung cancer cell migration and invasion. BMC Cancer. 2010; 10:364.
7. Malik G., Knowles LM, Dhir R, Xu S, Yang S, Ruoslahti E et al. Plasma fibronectin promotes lung metastasis by contributions to fibrin clots and tumor cell invasion. Cancer Res. 2010; 70:4327-34.
8. Roman J, Rizenthaler JD, Roser-Page S, Sun X, Han S. α5β1-integrin expression is essential for tumor progression in experimental lung cancer. Am J Respir Cell Mol Biol. 2010; 43:684-91.
9. Zheng Y, Ritzenthaler JD, Roman J, Han S. Nicotine stimulates human lung cancer cell growth by inducing fibronectin expression. Am J Respir Cell Mol Biol. 2007; 376:681-90.
10. Hartmann TN, Burger JA, Glodek A, Fujii N, Burger M. CXCR4 chemokine receptor and integrin signaling co-operate in mediating adhesion and chemoresistance in small cell lung cancer (SCLC) cells. Oncogene. 2005; 24:4462-71.
11. Liu F, Mih JD, Shea BS, Kho AT, Sharif AS, Tager AM et al. Feedback amplifications of fibrosis through matrix stiffening and COX-2 suppression. J Cell Biol. 2010; 190:693-708.
12. Levental KR, Yu H, Kass L, Lakins JN, Egeblad M, Erler JT et al. Matrix crosslinking forces tumor progression by enhancing integrin signaling. Cell. 2009; 139:891-906.
13. Antia M, Baneys G, Kubow KE, Vogel V. Fibronectin in aging extracellular matrix fibrils is progressively unfolded by cells and elicits an enhanced rigidity response. Farady Discuss. 2008; 139:229-49.
14. Gao M, Craig D, Lequin O, Campbell ID, Vogel V, Schulten K. Structure and functional significance of mechanically unfolded fibronectin type III1 intermediates. Proc Natl Acad Sci. 2003; 100:14784-9.
15. Klein RM, Zheng M, Ambesi A, van de Water L, McKeown-Longo PJ. Stimulation of extracellular matrix remodeling by the first type III repeat in fibronectin. J Cell Sci. 2003; 116:4663-74.
16. Gui L, Wojciechowski K, Gildner CD, Nedelkovska H, Hocking DC. Identification of the heparin-binding determinants within fibronectin repeat III1: role in cell spreading and growth. J Biol Chem. 2006; 281:34816-25.
17. Raman D, Baugher PJ, Thu YM, Richmond A. Role of chemokines in tumor growth. Cancer Lett. 2007; 256:137-65.
18. Jones CP, Pitchford SC, Lloyd CM, Rankin SM. CXCR2 mediates the recruitment of endothelial progenitor cells during allergic airways remodeling. Stem Cells. 2009; 27:3074-81.
19. Yi M, Ruoslahti E. A fibronectin fragment inhibits tumor growth, angiogenesis, and metastasis. Proc Natl Acad Sci. 2001; 98:620-4.
20. Ambesi A, Klein RM, Pumiglia KM, McKeown-Longo PJ. Anastellin, a fragment of the first type III repeat of fibronectin, inhibits extracellular signal-regulated kinase and causes G1 arrest in human microvessel endothelial cells. Cancer Res. 2005; 65:148-56.
21. Ambesi A, McKeown-Longo PJ. Anastellin, the angiostatic fibronectin peptide, is a selective inhibitor of lysophospholipid signaling. Mol Cancer Res. 2009; 7(2):255-65.
22. You R, Zheng M, McKeown-Longo PJ. The first type III repeat in fibronectin activates an inflammatory pathway in dermal fibroblasts. J Biol Chem. 2010; 285:36255-9.
23. Lorne E, Dupont H, Abraham E. Toll-like receptors 2 and 4: initiators of non-septic inflammation in critical care medicine? Inten Care Med. 2010; 36:1826-35.
24. Stewart CR, Stuart LM, Wilkinson K, van Gils JM, Deng J, Halle A et al. CD36 ligands promote sterile inflammation through assembly of a Toll-like receptor 4 and 6 heterodimer. Nat Immunol. 2010; 11:155-61.
25. Piccinini AM, Midwood KS. DAMPening inflammation by modulating TLR signalling. Mediators of Inflammation. 2010; Epub.